REMARKS

In the office action, the Examiner maintained the restriction requirement and raised various objections and rejections against the specification and the claims. Each objection and rejection raised by the Examiner is addressed separately below.

In view of the amendments noted above and remarks below, applicants respectfully request reconsideration of the merits of this patent application.

A petition for three months extension of time accompanies this response so that the response will be deemed to have been timely filed. If any other extension of time is required in this or any subsequent response, please consider this to be a petition for the appropriate extension and a request to charge the petition fee to Deposit Account No. 17-0055. No other fee is believed to be due in connection with this response. However, if any fee is due in this or any subsequent response, please charge the fee to the same Deposit Account No. 17-0055.

Restriction requirement

The Examiner maintained the restriction requirement and withdrew claims 6, 12, 13, and 22-36 from further consideration. Applicants respectfully submit that the subject matter of claims 12 and 13, which has been incorporated into amended claim 1 (claims 12 and 13 themselves are canceled), should have been examined because they read on elected invention I(A). As shown in Example 8 and Fig. 11C of the application, HET0016 is an inhibitor of the enzymes recited in claims 12 and 13.

Applicants further respectfully note that "Schimidt et al., 2000" referred to at page 2, line 13 of the office action should be "Su et al. 1999." Applicants do not agree with the Examiner that Su et al. 1999 defeat the shared technical feature of Groups I and II for the reasons set forth below in connection with the 35 U.S.C. §102 (b) rejections.

Objections to the specification

The Examiner objected to the title of the application for not being descriptive of the elected invention, i.e., a method of treatment using HET0016. Applicants respectfully traverse the objection. As provided in the restriction requirement mailed April 19, 2005, claims 1-3 were designated as linking claims and the restriction requirement is subject to the nonallowance of the linking claims. Under MPEP 809, "the linking claims must be examined with, and thus are considered part of, the invention elected." Linking claims 1-3 are not limited to HET0016. For example, the Examiner examined claims 1-5 and 17 in connection with dibromododecenyl methylsulfimide and 1-aminobenzotriazole (see rejections under 35

U.S.C. §102 in the office action). Therefore, applicants respectfully submit that the title of the application is appropriate.

The Examiner further objected to the abstract for not being provided on a separate sheet. Applicants note that when the corresponding PCT application was filed, a separate sheet containing the abstract was submitted. Applicants herein resubmit the separate sheet containing the abstract.

Objections to the claims

The Examiner objected to the claim set for not beginning with a sentence of which the claims are an object. Applicants respectfully traverse the objection. As provided on page 21 of the application, the claim set begins with the sentence "WE CLAIM..." of which the claims are an object.

The Examiner next objected to claims 1-5 and 14-18 for reciting non-elected subject matter. Claims 2-5, 14, 16, and 18 have been canceled. Applicants respectfully traverse the objection in connection with remaining claims 1, 15, and 17. As discussed above, under MPEP 809, "the linking claims must be examined with, and thus are considered part of, the invention elected." Applicants respectfully submit that claims 1, 15, and 17 are linking claims for the elected subject matter of Group I(A), i.e., a method of treatment using a 20-HETE synthesizing enzyme inhibitor HET0016. As an example, the Examiner examined claims 1-5 and 17 in connection with not only HET0016 but also dibromododecenyl methylsulfimide and 1-aminobenzotriazole (see rejections under 35 U.S.C. §102 in the office action). Therefore, claims 1, 15, and 17 are appropriate in form.

The Examiner next objected to claims 2, 3, and 5 for improper Markush language. Claims 2, 3, and 5 have been canceled. This objection is moot.

The Examiner next objected to claims 8, 9, 11, and 21 alleging that the term "the dose" recited in the claims lack antecedent basis. Claim 21 has been canceled and claims 8, 9, and 11 have been amended to recite "a dose." The objection is believed to have been overcome.

Claim rejections under 35 U.S.C. §101

The Examiner rejected process claims 1-3 and 18 alleging that they do not set forth any steps involve in the process. While not agreeing with the Examiner, claim 1 is amended to recite an administering step for other reasons and the rejection is rendered moot.

The Examiner next provisionally rejected claims 1-5, 7-11, and 14-21 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-4, 6-13, 19-22, and 24-28 of U.S. Application No. 10/986,695. Applicants stand ready to address the rejection should it be maintained as an actual rejection.

Claim rejections under 35 U.S.C. §112-second paragraph

The Examiner rejected claims 1-5, 7-11, and 14-18 as being indefinite. With respect to method claims 1-3 and 18 in particular, the Examiner alleged that they do not set forth any steps involved in the method. While not agreeing with the Examiner, claim 1 has been amended to recite an administering step for other reasons and the rejection is rendered moot.

With respect to claim 4 and its dependents (claims 5, 7-11, and 14-17), the Examiner alleged that it is not clear whether the treatment of a human or non-human animal is intended. Claims 4, 5, 14, and 16 have been canceled and claims 7-11, 15, and 17 have amended to depend on new claim 39. This rejection is moot.

Enablement rejection under 35 U.S.C. §112-first paragraph

The Examiner rejected claims 1-5, 7-11, and 14-21 for failing to meet the enablement requirement. In particular, the Examiner alleged that the specification, while being enabling for intravenous administration of HET0016 to increase cerebral blood following after subarachnoid hemorrhage and to decrease infarct volume flowing transient occlusion of the middle cerebral artery, does not enable all methods of treating any cerebral vascular disease in any animal using any compound that decreases the activity of any 20-HETE synthesizing enzyme.

While not agreeing with the Examiner, claim 1 has been amended to facilitate prosecution. Applicants reserve the right to pursue canceled subject matter in a subsequent application.

Claim 1 as amended is directed at a method for treating a cerebral vascular disease in a human or non-human animal wherein the cerebral vascular disease is selected from the group consisting of occlusive stroke, hemorrhagic stroke, cerebrovasospasm associated with hemorrhagic stroke, and accumulation of blood in subarachnoid space caused by head injury. The method involves administering into the human or non-human animal an inhibitor of a 20-HETE synthesizing enzyme selected from the group consisting of a cytochrome P450 4A (CYP4A) enzyme and a cytochrome P450 4F (CYP4F) enzyme in an amount sufficient to

increase or prevent a decrease in cerebral blood flow in the human or non-human animal. Applicants respectfully submit that claim 1 and its dependents as amended are enabled.

Amended claim 1 and its dependents are limited to four particular cerebral vascular diseases. The treatment of these four diseases are enabled by the examples provided in the application. For example, in example 1, autologous arterial blood was injected into the Cisterna Magna of Sprague-Dawley rats to model hemorrhagic stroke and accumulation of blood in subarachnoid space caused by head injury. The injection led to lowered cerebral blood flow reflecting cerebrovasospasm associated with hemorrhagic stroke. In example 2, occlusive (ischemic) stroke was modeled in Wistar rats by the insertion of a silicone-coated suture from the lumen of the right external carotid artery to the right internal carotid artery to block the origin of the right middle cerebral artery. In both examples, 20-HETE synthesizing enzyme inhibitors were shown to be able to treat the diseases.

With regard to the scope of the animals that are enabled, applicants first note that rats have been used to study subarachnoid hemorrhage for many many years (see e.g., Delgado TJ et al., Stroke 16:595-602, 1985; and Solomon RA et al., Stroke 16:58-64, 1985) and it is well understood in the art that rats can be used to study human diseases. Based on the rat data provided in examples 1 and 2, it is reasonable to expect that 20-HETE synthesizing enzyme inhibitors will also help human patients suffering from the same diseases.

For other non-human animals, the Examiner has not provided any evidence that suggests that 20-HETE inhibitors will not work in them. In fact, it is well known in the art that 20-HETE and its function are conserved across animal species. Accordingly, it is reasonable to expect that 20-HETE inhibitors will work in other animals.

With regard to the routes of administration that are enabled, applicants first note that examples 1 and 2 and paragraphs [0026] and [0027] of the application specifically teach intravenous injection, injection to a hemorrhage site in the brain, and injection into the cerebrospinal fluid. Therefore, these routes of administration are enabled.

Furthermore, applicants respectfully submit that those pending claims not limited by a specific route of administration are also enabled. The Examiner has not provided any evidence to suggest that a particular route of administration does not work. The evidence is to the contrary. All routes that have been tested by applicants as provided in the application worked. Even if it is scientifically sound to doubt the other, non-exemplified administrative routes, the claimed invention is nevertheless enabled because the claims may encompass inoperable subject matter. In this regard, the enablement standard is whether a skilled person could determine which embodiments that were conceived, but not yet made, would be

inoperative or operative with expenditure of no more effort than is normally required in the art. See MPEP 2164.08(b), citing *Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 750 F.2d 1569, 1577, 224 USPQ 409, 414 (Fed. Cir. 1984) (prophetic examples do not make the disclosure nonenabling). If the experimentation involved is merely routine, a considerable amount is permissible. See *In re Wands*, 858 F. 2d 731 (Fed. Cir. 1988). Applicants respectfully submit that a skilled artisan can simply administer a 20-HETE synthesizing enzyme inhibitor such as HET0016 via a particular route using one of the well known rat models provided in the application and will be able to determine whether the route is effective. Such an experiment is merely routine. Therefore no more effort than that is normally required is involved in the determination of the effectiveness of a particular route of administration.

With regard to 20-HETE synthesizing enzymes, although inhibitors of all 20-HETE synthesizing enzymes are believed to have been enabled, claim 1 has been amended to limit the 20-HETE synthesizing enzymes to a CYP4A or CYP4F enzyme to facilitate prosecution. It is well established in the art that enzymes of the CYP4A and CYP4F families catalyze the synthesis of 20-HETE (see paragraph [0005] on page 2 of the application). Using CYP4A11, CYP4F2, and CYP4F3 as examples, applicants have shown HET0016 inhibits the activity of CYP4A and CYP4F enzymes. Therefore, claim 1 and its dependents as amended are enabled with respect to the use of a CYP4A or CYP4F inhibitor.

With regard to 20-HETE synthesizing inhibitors, the Examiner alleged that the specification fails to establish the structure of all compounds that are inhibitors of any 20-HETE synthesizing enzyme and the structure of inhibitors of any 20-HETE synthesizing enzyme that can successfully treat any cerebral vascular disease (page 10, (C) and (E)). In addition, the Examiner alleged that the specification fails to establish how any of the inhibitors can or cannot be modified and still inhibit a 20-HETE synthesizing enzyme or successfully be used to treat any cerebral vascular disease (page 10, (D) and (F)). Furthermore, the Examiner alleged that the specification fails to establish a rational and predictable scheme for modifying any inhibitor compound with an expectation of obtaining the desired treatment result (page 10, (H)). Applicants respectfully traverse the enablement rejection in connection with the above allegations.

Applicants first note that claim 1 and its dependents have been amended to recite four particular cerebral vascular diseases and a CYP4A or CYP4F enzyme. In this regard, the gist of the invention resides in the recognition that the diseases are associated an increase in the 20-HETE level and the demonstration that 20-HETE synthesizing enzyme inhibitors

HET0016 and 17-ODYA are effective in treating the diseases. From this, a skilled artisan appreciates that other 20-HETE synthesizing enzyme inhibitors can also be used to treat the diseases.

It is noted that identifying new 20-HETE synthesizing enzyme inhibitors either by testing new compounds or modifying existing inhibitors is not part of the invention. The present invention only calls for the use of known inhibitors or those that will become known, independent of the present invention, in the future. The application provides numerous examples of known 20-HETE synthesizing enzyme inhibitors (e.g., HET0016, 17-ODYA, dibromododecenyl methylsulfimide, 1-aminobenzotriazole, and miconazole) and a skilled artisan is familiar with others. A skilled artisan will certainly become familiar with any new 20-HETE synthesizing inhibitors once they are identified. When an enzyme and inhibitors of the enzyme are well known in the art, one or more examples provided in the specification has been deemed adequate to support the use of an inhibitor in general under the U.S. patent law. For example, in *In re Herschler*, 591 F.2d 693 (CCPA 1979), the court found adequate support for broad claims to processes for topically administering a physiologically active steroidal agent to a human or animal by concurrently administering the steroidal agent and DMSO, even though the specification disclosed only one example of a "physiologically active steroidal agent" because numerous physiologically active steroidal agents were known to the skilled artisan. This holding is recently upheld by the Federal Circuit in *University of Rochester v. G.D. Searle & Co., Inc.*, 358 F.3d 916, 928 (Fed. Cir. 2004). With regard to 20-HETE synthesizing enzyme inhibitors not yet identified, the Federal Circuit has held that the enablement requirement is not applicable to future technologies. *Chiron Corporation v. Genentech Inc.*, 363 F.3d 1247 (Fed. Cir. 2004). Consistent with the above case law, the U.S. Patent and Trademark Office considers a claim that involves the use of any inhibitor to an enzyme patentable if a few examples are provided in the specification. For example, in each of U.S. Patent Nos. 6,756,399; 6,455,541; 6,376,528; 6,191,169; 6,187,756; 6,136,839; and 5,928,654, claims directed at the use of any inhibitor of a particular enzyme were allowed even though the specification names only a few examples. Therefore, the use of a CYP4A or CYP4F inhibitor in general is enabled.

For all of the above reasons, applicants respectfully submit and claim 1 and its dependents as amended satisfy the enablement requirement.

Written description rejection under 35 U.S.C. §112-first paragraph

The Examiner rejected claims 1-5, 7-11, and 14-21 for failing to satisfy the written description requirement. In particular, the Examiner alleged that while the claims at issue are directed to a genus of methods for treating any cerebral vascular disease in any animal using any compound that decreases the activity of any 20-HETE synthesizing enzyme, the specification teaches only two representative species of such methods.

While not agreeing with the Examiner, claim 1 has been amended to facilitate prosecution. As discussed above in connection with the enablement requirement, the application clearly conveys to one skilled in the art through the description and the examples of the application that CYP4A and CYP4F inhibitors can be used to treat the four specific cerebral vascular diseases recited in amended claim 1. Also as discussed above, one skilled in the art understands that the structure and function of 20-HETE are conserved across animal species and it is reasonable to extrapolate the observations made with rats to humans as well as other animals. Further as discussed above, one skilled in the art would understand from the application that the invention is not limited to a specific route of administration or a specific CYP4A or CYP4F inhibitor. Therefore, claim 1 and its dependents, as amended, satisfy the written description requirement.

Claim rejections under 35 U.S.C. §102

1. Alonso-Galicia et al. 1999

The Examiner rejected claims 1-5 and 17 as being anticipated by Alonso-Galicia et al. 1999, alleging that the reference teaches that intracerebroventricular injection of dibromododecenyl methylsulfimide reduces cerebral blood flow (Fig. 7) and thereby anticipates claims 1-5 and 17. Claim 1 has been amended, claims 2-5 have been canceled, and new claims 37-43 have been added. Applicants respectfully traverse the rejection in connection with claims 1 and 17 as well as any new claim that the Examiner may deem relevant.

As the Examiner correctly pointed out, Alonso-Galicia et al. 1999 teach that intracerebroventricular injection of dibromododecenyl methylsulfimide reduces cerebral blood flow increase (caused by the short acting NO donor MAHMA nonoate). However, the pending claims involve the use of a 20-HETE synthesizing enzyme inhibitor to increase or prevent a decrease in cerebral blood flow. Therefore, Alonso-Galicia et al. 1999 do not anticipate the pending claims.

2. Su et al. 1999

The Examiner rejected claims 1-5 as being anticipated by Su et al. 1999 as evidenced by Fotherby et al. 1997 or Schmidt et al. 2000. In particular, the Examiner alleged that Su et al. 1999 teach that 1-aminobenzotriazole inhibits 20-HETE synthesis (Fig. 1) and reduces blood pressure (Fig. 9) while Fotherby et al. and Schmidt et al. demonstrate that it is well known in the art that reducing blood pressure is an effective way for treating cerebral vascular diseases. Claim 1 has been amended, claims 2-5 have been canceled, and new claims 37-43 have been added. Applicants respectfully traverse the rejection in connection with claim 1 and any new claim that the Examiner may deem relevant.

Su et al. 1999 teach that 1-aminobenzotriazole is a 20-HETE inhibitor and it was able to lower blood pressure in a strain of <u>spontaneously hypertensive rats</u> (SHR). However, Su et al. 1999 do not show whether 1-aminobenzotriazole can lower blood pressure in <u>normotensive</u> rats. In fact, in subsequent studies with normotensive rats, others have shown that 1-aminobenzotriazole caused hypertension when the rats were fed high salt diet and lowered blood pressure when the rats were fed low salt diet (Hoagland et al., Hypertension 42:669-673, 2003, copy attached). In the same normotensive rats, HET0016 either caused hypertension or did not change the blood pressure (Hoagland et al., Hypertension 42:669-673, 2003).

Furthermore, it is not true that lowering blood pressure is an effective treatment for occlusive stroke and hemorrhagic stroke. Fotherby et al. and Schmidt et al. teach that chronic hypertension increases the risk for stroke by promoting arteriolosclerosis and narrowing the cerebral arteries. However, they do not establish that lowering blood pressure is an effective treatment for stroke. Fotherby et al. noted that "[t]he risks and benefits of antihypertensive therapy in the large number of older and frailer stroke patients commonly seen in our hospital remain largely unresolved" (page 625, right column, lines 9-12). Schmidt et al. only mention that hypertension is associated with stroke but do not provide that lowering blood pressure is an effective treatment. In fact, lowering blood pressure with systemic vasodilators or antihypertensive drugs are counterindicated in the treatment of both occlusive and hemorrhagic stroke. In both instances the goal is to increase blood flow to vessels that are occluded (occlusive stroke) or leaking (hemorrhagic stroke). In the case of occlusive stroke, flow can be restored by raising systemic pressure to the bed to push flow through the occlusion or increase collateral flow. Current treatment emphasizes triple H therapy, i.e., hypervolemia, <u>hypertension</u>, and hemodilation to maintain blood flow to the at risk region of the brain (Treggiari MM et al. J. Neurosurg. 99:978-984, 2003). Volume expansion is used

to enhance cardiac output and reduce vasoconstriction. Hemodilution reduces the viscosity of the blood and hypertension driven by inotrophic drugs like dobutamine is used to maintain adequate perfusion.

For the above reasons, Su et al. 1999 together with Fotherby et al. and Schmidt et al. do not anticipate the pending claims.

Claim rejections under 35 U.S.C. §103

The Examiner rejected claims 1-5 as being obvious over Roman et al. 1999 (WO 99/43310) in view of Frisbee et al. 2000. In particular, the Examiner alleged that Roman et al. disclosed that cerebral vascular diseases can be treated by blocking the effect of 20-HETE and Frisbee et al. disclosed that 17-ODYA and dibromododecenyl methylsulfimide inhibit 20-HETE production. Claim 1 has been amended, claims 2-5 have been canceled, and new claims 37-43 have been added. Applicants respectfully traverse the rejection in connection with claim 1 and any new claim that the Examiner may deem relevant.

Roman et al. 1999 disclosed the use of 20-HETE antagonists to treat cerebral vascular diseases (page 5, line 4). 20-HETE antagonists block the 20-HETE receptor while 20-HETE synthesizing enzyme inhibitors inhibit the synthesis of 20-HETE. As shown in the application, hemorrhagic stroke is associated with an elevation in the formation of 20-HETE in the cerebral circulation and that a 20-HETE synthesizing enzyme inhibitor could be given to treat the acute fall in cerebral blood flow and the vasospasm caused by hemorrhagic stroke. The same thing is true for occlusive stroke. The levels of 20-HETE in the brain can increase because of increased synthesis or the release of preformed 20-HETE stored in membrane phospholipid pools in (i) blood elements such as white blood cells and platelets, (ii) neural tissues such as brain neurons, (iii) vascular tissues such as vascular myocytes, and (iv) circulates bound to plasma proteins. While a 20-HETE antagonist is effective regardless of what causes the increase in 20-HETE level because it blocks the downstream 20-HETE receptor, a 20-HETE synthesis inhibitor will not be effective if 20-HETE is released from storage pools. Neither Roman et al. 1999 nor Frisbee et al. disclose or suggest that the increase in 20-HETE level in hemorrhagic or occlusive stroke is caused by increased synthesis but not release from storage pools. Therefore, it is not obvious that a 20-HETE synthesizing enzyme inhibitor will work. At the most, the present invention might be merely obvious to try, but without reasonable likelihood of success.

Conclusion

Having addressed each issue raised by the Examiner, claims 1, 7-11, 15, and 17 as amended and new claims 37-43 are believed to be in condition for allowance and a Notice of Allowance is respectfully requested. Should any issues remain outstanding, the Examiner is invited to contact the undersigned at the telephone number appearing below if such would advance the prosecution of this application.

Respectfully submitted,

Zhibin Ren
Reg. No. 47,897
Attorney for Applicants
QUARLES & BRADY LLP
411 East Wisconsin Avenue
Milwaukee, WI 53202-4497
TEL (414) 277-5633
FAX (414) 271-3552

QBMKE\5820326.2

State of the Art Lecture

Inhibitors of 20-HETE Formation Promote Salt-Sensitive Hypertension in Rats

Kimberly M. Hoagland, Averia K. Flasch, Richard J. Roman

Abstract—This study examined whether chronic blockade of epoxyeicosatrienoic acids (EETs) and/or 20-hydroxyeicosatetraenoic acid (20-HETE) formation promotes development of salt-sensitive hypertension. Changes in blood pressure, renal cytochrome P450 metabolism of arachidonic acid, and 20-HETE excretion in response to a high salt diet were measured in rats chronically treated with 1-aminobenzotriazole (ABT, 50 mg/kg per day) to block EETs and 20-HETE formation or *N*-hydroxy-*N*′-(4-butyl-2 methylphenyl) formamidine (HET0016, 10 mg/kg per day) that selectively reduces 20-HETE formation. ABT reduced blood pressure in rats fed a low salt (0.4% NaCl) diet, but blood pressure rose by 20 mm Hg after these rats were switched to a high salt (8% NaCl) diet for 10 days. HET0016 had no effect on blood pressure in rats fed a low salt diet; however, blood pressure rose by 18 mm Hg after the rats were fed a high salt diet. 20-HETE formation in kidney homogenates rose by 30% and epoxygenase activity doubled when rats were fed a high salt diet. Chronic treatment with ABT and HET0016 inhibited the renal formation of 20-HETE by ≈90%. Renal epoxygenase activity decreased by 76% in ABT-treated rats and was not significantly altered in rats treated with HET0016. 20-HETE excretion rose from 470±21 to 570±41 ng/d when the rats were switched from the low to the high salt diet. 20-HETE excretion fell by 68% and 85% in rats that were chronically treated with ABT and HET0016. These results suggest that chronic blockade of the formation of 20-HETE promotes the development of salt-sensitive hypertension in rats. (*Hypertension*. **2003;42[part 2]:669-673.**)

Key Words: rats, Dahl ■ metabolism ■ arachidonic acids ■ blood pressure ■ hypertension, sodium-dependent

The role of the cytochrome P450 (P450) metabolites of arachidonic acid (AA) in the development and maintenance of hypertension remains uncertain in part because this pathway has both prohypertensive and antihypertensive actions.[1] 20-Hydroxyeicosatetraenoic acid (20-HETE) inhibits Na^+ transport in the proximal tubule and thick ascending limb of the loop of Henle (TALH),[2–5] and compounds that induce the renal formation of 20-HETE lower blood pressure in Dahl salt-sensitive (DS) rats.[1] Similarly, epoxyeicosatrienoic acids (EETs) inhibit Na^+ transport in the proximal tubule and collecting duct,[6,7] and increasing the renal levels of EETs with inhibitors of soluble epoxide hydrolase (sEH) reduces blood pressure in spontaneously hypertensive rats (SHRs)[8] and angiotensin II–induced hypertensive rats.[9] However, 20-HETE is also a potent vasoconstrictor,[1] and many investigators have reported that blocking the vascular effects of 20-HETE may contribute to its antihypertensive effect in SHR and other experimental models of hypertension.[10,11]

Part of the problem in defining the role of the P450 metabolites of AA in the control of blood pressure has been the lack of selective inhibitors of the pathway that chronically block the formation of 20-HETE and EETs in vivo. Inhibitors that are commonly used to inhibit the formation of EETs and 20-HETE in vitro, such as 17-octadecynoic acid (17-ODYA), dibromododecenyl methylsulfimide (DDMS), and methylsuphonyl-6-(2-propargyloxy-phenyl)-hexanamide (PPOMS), bind to plasma proteins, and in our hands are not effective in reducing the renal formation of EETs and 20-HETE after systemic administration. More recently, 1-aminobenzotriazole (ABT) has been reported to reduce the renal formation of 20-HETE and/or EETs when given acutely to rats,[10,12,13] and *N*-hydroxy-*N*′-(4-butyl-2 methylphenyl) formamidine (HET0016) has been shown to be the most selective inhibitor of the renal formation of 20-HETE.[14] Therefore, in the current study, we examined the effects of chronic administration of ABT and HET0016 on the renal metabolism of AA, the urinary excretion of 20-HETE, and blood pressure in Sprague-Dawley (SD) rats fed a low or a high salt diet.

Methods

Animals

Experiments were performed in adult male SD rats weighing between 300 and 350 g purchased from Harlan (Indianapolis, Ind). The rats were housed in stainless steel metabolic cages in a chronic monitoring facility at the Medical College of Wisconsin, approved by the American Association for the Accreditation of Laboratory Animal Care. The rats were fed a purified diet (AIN-76 A) purchased from Dyets, Inc, that contained either 0.4% (cat no. 113755) or 8%

Received May 5, 2003; first decision May 14, 2003; revision accepted June 23, 2003.
From the Department of Physiology, Medical College of Wisconsin, Milwaukee.
Correspondence to Richard J. Roman, PhD, Medical College of Wisconsin, Department of Physiology, 8701 Watertown Plank Road, Milwaukee, WI 53226. E-mail rroman@mcw.edu

Hypertension is available at http://www.hypertensionaha.org DOI: 10.1161/01.HYP.0000084634.97353.1A

NaCl (cat no. 100078) by weight. The rats had free access to food and water throughout the study. All protocols were approved by the Animal Welfare Committee at the Medical College of Wisconsin and were in accordance with the National Institute of Health's "Guide for the Care and Use of Laboratory Animals."

Surgical Preparation of Animals for Chronic Study

The rats were anesthetized with an intramuscular injection of ketamine (40 mg/kg), xylazine (2.5 mg/kg), and acepromazine (0.6 mg/kg). Microrenathane (Braintree Scientific, Inc) catheters were chronically implanted into the left femoral artery and vein for measurement of mean arterial pressure (MAP) and intravenous drug administration. The catheters were tunneled subcutaneously, exteriorized between the scapulae, and protected by a stainless steel spring attached to a dual-channel swivel device anchored above the cage so that blood pressure could be continuously monitored in conscious, unrestrained rats. After surgery, the rats received enrofloxacin (20 mg/kg) to prevent infection. The catheters were flushed daily with 0.3 mL isotonic saline containing heparin (500 U/mL).

Experimental Protocol

The rats were maintained on a low salt diet containing 0.4% NaCl for 5 days to recover from surgery. Baseline MAP was then recorded on 3 consecutive control days while the rats remained on the low salt diet. The arterial catheters were connected to transducers interfaced with a computerized data acquisition system, and systolic, diastolic, and mean arterial blood pressure and heart rate were continuously recorded at a frequency of 300 Hz for 5 hours per day between 10:00 AM and 3:00 PM. MAP was averaged over 1-minute intervals, and a single value was determined for each recording session. The rats were divided into 4 treatment groups and received either ABT (50 mg/kg per day IV, n=5) to block the renal formation of EETs and 20-HETE,[10,12] HET0016 (10 mg/kg per day IV, n=7) to selectively inhibit the formation of 20-HETE,[14,15] or the vehicles for ABT (0.9% NaCl, n=5) and HET0016 (10% lecithin in 0.9% NaCl solution, n=4). After administration of the P450 inhibitors or the vehicles, the rats remained on the low salt diet and MAP was recorded for 2 days. Then, the rats were switched to a high salt (8% NaCl) diet and MAP was recorded for an additional 10 days. Other experiments were performed in rats treated with ABT (n=5) and HET0016 (n=5) that were maintained on the low salt diet throughout the experiment to determine whether the blood pressure responses to these inhibitors were salt-dependent.

Urine samples were collected during the control period and on day 10 of the high salt diet for measurement of 20-HETE excretion. The samples were collected into glass bottles placed on dry ice that contained 5 mg triphenylphosphine to prevent oxidation of 20-HETE. The concentration of 20-HETE in the urine samples was determined through the use of a fluorescent high-performance liquid chromatography (HPLC) assay, as previously described.[12]

Renal P450 AA Metabolism Assay

The effects of chronic treatment of rats with ABT and HET0016 on renal metabolism of AA by renal cortical homogenates were studied. Crude homogenates, instead of renal microsomes, were used in these experiments to avoid dilution of the tissue levels of the drugs, since HET0016 is a reversible competitive inhibitor of the formation of 20-HETE. In these experiments, the chronically treated rats were anesthetized with sodium pentobarbital (50 mg/kg IV), and the kidneys were removed. The kidneys were homogenized in 1 mL of a 10-mmol/L potassium buffer (pH 7.7) containing 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 7.5 μL/mL protease inhibitor cocktail (Sigma, cat no. P-8340). The homogenates were centrifuged for 15 minutes at 3000g and for 30 minutes at 11 000g. The protein concentrations of the homogenates were determined with the use of the Bradford method with bovine γ-globulin as a standard. P450 enzyme activity was determined by incubating samples (1 mg protein) with [^{14}C]-AA (0.2 μCi, 1.8 μmol/L) in 0.5 mL of a 100-mmol/L potassium phosphate buffer (pH 7.4) containing 10 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L NADPH, and an NADPH-regenerating system (10 mmol/L isocitrate and 0.16 U/mL isocitrate dehydrogenase) at 37°C for 15 minutes. These reactions were run without adding cold AA because it competes with the competitive inhibitor HET0016. Acidic lipids were extracted with ethyl acetate and dried under N_2. Metabolites were separated by using a 2×250-mm C18-reverse-phase HPLC column (Supelco Co Inc) and a linear elution gradient ranging from acetonitrile/water/acetic acid (50/50/0.1, vol/vol/vol) to acetonitrile/acetic acid (100/0.1, vol/vol) over a 40-minute period. Epoxygenase activity was reported as the sum of the formation of EETs and dihydroxyeicosatrienoic acids (DiHETEs).

Statistical Analysis

Values are presented as mean±SEM. Significance of differences between mean values were determined with the use of an ANOVA followed by the Student-Newman-Keuls post hoc test. A value of $P<0.05$ for a 2-tailed test was considered statistically significant.

Results

Effects of ABT and HET0016 Treatment on Blood Pressure

The effects of chronic blockade of the renal formation of EETs and 20-HETE with ABT or 20-HETE alone with HET0016 on MAP are summarized in Figure 1. Since there were no differences in MAP in the rats that received the different vehicles for HET0016 and ABT, the data from these two control groups were combined. Blood pressure did not change during the protocol in the vehicle-treated rats. In rats fed a low salt diet, MAP fell by ≈10 mm Hg on the first day of ABT treatment (Figure 1A) and remained significantly below control throughout the study. In contrast, HET0016 had no effect on MAP in rats fed a low salt diet (Figure 1B). MAP gradually increased by ≈20 mm Hg in both the ABT-treated and HET0016-treated rats when the rats were switched to a high salt diet for 10 days. In contrast, MAP did not increase in rats treated with ABT or HET0016 that were maintained on a low salt diet throughout the study.

Effect of ABT and HET0016 Treatment on Renal Metabolism of AA

The effects of ABT and HET0016 on the formation of 20-HETE, EETs, and DiHETEs in renal homogenates are presented in Figure 2A. ABT and HET0016 reduced the synthesis of 20-HETE by ≈90% in rats fed either a low or a high salt diet. ABT also reduced cortical epoxygenase activity by 50% in the rats maintained on a low salt diet and by 76% in the rats fed high salt diet for 10 days. However, epoxygenase activity was not significantly altered in rats that were treated with HET0016.

Effect of ABT and HET0016 Treatment on Urinary Excretion of 20-HETE

The effects of ABT and HET0016 on the excretion of 20-HETE are summarized in Figure 2B. The excretion of 20-HETE averaged 470±21 ng/d when the rats were fed a low salt diet. 20-HETE excretion increased by 20% when rats were fed a high salt diet for 10 days. ABT and HET0016 treatment reduced 20-HETE excretion by ≈70% and ≈90%, respectively.

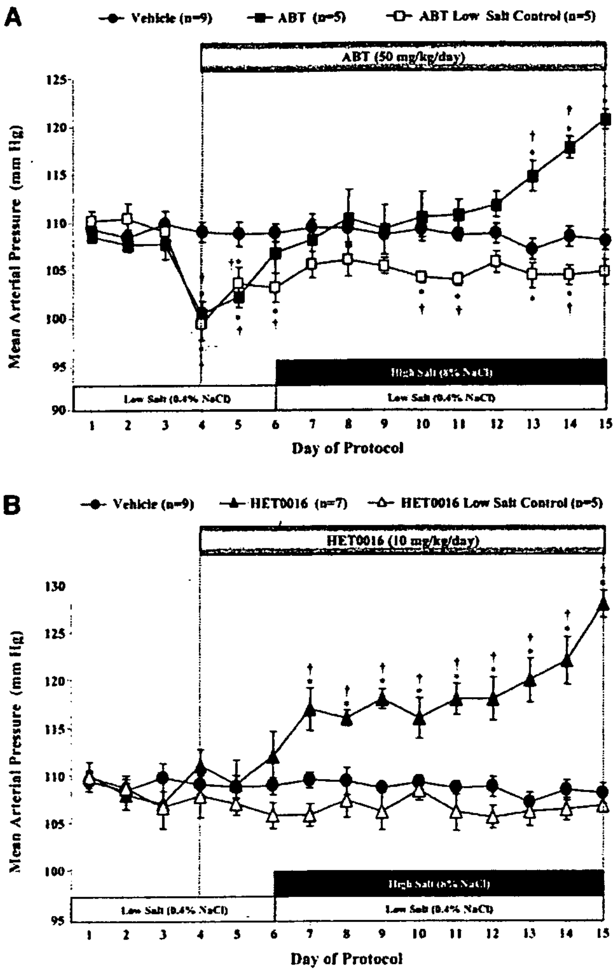

Figure 1. Effects of blockade of P450 metabolism of AA on blood pressure in SD rats fed a high salt diet. Blood pressure was recorded from conscious SD rats chronically treated with either ABT (A) to block the formation of EETs and 20-HETE or HET0016 (B) to selectively block the formation of 20-HETE. Mean±SEM values are presented. Numbers in parentheses indicate number of rats studied in each group. *$P<0.05$ vs baseline blood pressure. †$P<0.05$ vs corresponding value in vehicle-treated rats.

Discussion

In the current study, ABT reduced blood pressure by ≈10 mm Hg in SD rats fed a low salt diet. This fall in MAP is consistent with previous findings in the SHR, in which inhibition of the renal formation of 20-HETE with ABT,[13] sodium 10-undecynyl sulfate (10-SUYS),[11] or antisense oligonucleotides[16] have been reported to reduce blood pressure in this strain. ABT has also been reported to lower blood pressure in deoxycorticosterone acetate (DOCA) salt–hypertensive rats[17,18] and in rats with angiotensin II–induced hypertension.[10,19]

In contrast, HET0016 (10 mg/kg per day) had no effect on blood pressure in the SD rats fed a low salt diet, despite the fact that HET0016 is a more potent and selective inhibitor of the formation of 20-HETE than ABT. We also found that this dose of HET0016 was effective and reduced both the formation of 20-HETE in renal cortical homogenates and the urinary excretion of 20-HETE by ≈90%. Thus, the reason for the differences in the blood pressure response in rats fed a low salt diet that were treated with ABT and HET0016 is not clear. Findings from the current study as well as previous studies[10,12] indicate that ABT blocks the formation of both EETs and 20-HETE in the kidney after chronic administration. This would suggest that the difference in the blood pressure response seen in rats treated with HET0016 and ABT may be due to an effect of ABT to inhibit formation of EETs. However, this hypothesis is not consistent with previous reports demonstrating that EETs are potent vasodilators, promote natriuresis, and have antihypertensive properties.[8,9] This may indicate that the reduction in blood pressure after ABT administration is mediated by some action that is independent of blockade of the formation of EETs and/or 20-HETE. In support of this view, other investigators have demonstrated that ABT does inhibit the activity of several other P450 enzymes besides those that produce EETs and 20-HETE.[13,20,21]

The current study also compared the effects of chronic blockade of the renal formation of 20-HETE with ABT or HET0016 on blood pressure in SD rats fed a high salt diet. MAP gradually rose by 20 mm Hg over a 10-day period in rats that were treated with ABT or HET0016. In contrast, blood pressure was not significantly altered in the SD rats fed

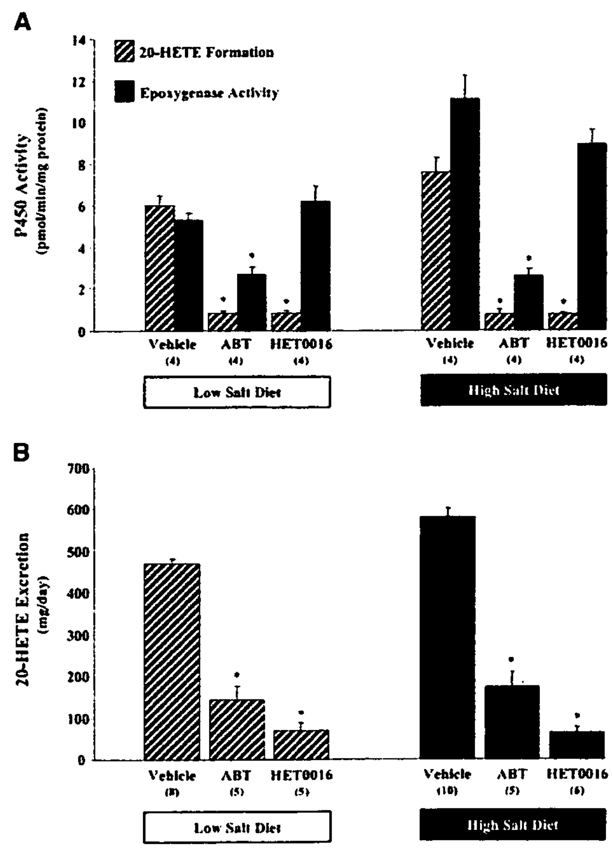

Figure 2. Effect of ABT and HET0016 on renal metabolism of AA (A) and urinary excretion of 20-HETE (B) in SD rats fed a low and/or high salt diet. Epoxygenase activity indicates EETs+DiHETEs formation. Mean±SEM values are presented. Numbers in parentheses indicate number of rats studied in each group. *$P<0.05$ vs vehicle-treated rats.

BEST AVAILABLE COPY

a high salt diet that were treated with vehicle or in rats treated with ABT and HET0016 that were maintained on a low salt diet throughout the study. Both ABT and HET0016 were equally effective in blocking the formation of 20-HETE in the kidney and in reducing the urinary excretion of 20-HETE. Taken together, these findings suggest that chronic blockade of the renal formation of 20-HETE promotes the development of salt-sensitive hypertension in otherwise salt-insensitive SD rats. This finding is consistent with previous studies, indicating that a deficiency in the renal formation of 20-HETE[3,22] plays a critical role in elevating loop Cl^- transport, resetting pressure natriuresis and the development of hypertension in DS rats.[3,5] Our findings are also consistent with those of Stec et al,[23] who demonstrated that chronic blockade of the renal formation of 20-HETE with an intrarenal infusion of 17-ODYA induced salt-sensitive hypertension in Lewis rats; it also fits with a recent report by Laffer et al[24]showing that 20-HETE excretion is linked to salt sensitivity of blood pressure in humans.

The finding that a reduction in the renal formation of 20-HETE is associated with the development of salt-sensitive hypertension in normally salt-resistant SD rats suggests that 20-HETE may contribute to the renal adaptation to elevations in Na^+ intake. However, the current results indicate that elevations in salt intake have little effect on the renal production or excretion of 20-HETE in SD rats. These results are consistent with previous reports showing that the renal formation of 20-HETE either did not change or fell when Lewis, Brown-Norway, SD, DR, and DS rats are fed a high salt diet.[22,25–28] Together, these findings indicate that 20-HETE in the kidney plays an important role in the chronic regulation of Na^+ excretion and blood pressure, but elevations in renal 20-HETE production per se do not contribute to the inhibition of Na^+ transport associated with elevations in salt intake.

Renal epoxygenase activity increased in rats fed a high salt diet in the current study. This finding confirms earlier reports by Makita and colleagues[29] and Holla and coworkers,[30] suggesting that epoxygenase activity is influenced by salt intake and that an inability to upregulate epoxygenase activity may contribute to the development of hypertension in DS rats. Our findings also are consistent with recent findings that administration of inhibitors of sEH, to elevate the renal formation of EETs, lowers blood pressure in SHRs[8] and attenuates angiotensin II–induced hypertension.[9] However, in the current study, there was no difference in the salt sensitivity of blood pressure in SD rats treated with either ABT or HET0016, even though renal epoxygenase activity was inhibited in rats treated with ABT but not in rats treated with HET0016. This finding suggests that it is likely that the fall in renal 20-HETE levels rather than the levels of EETs determines salt sensitivity of blood pressure in rats.

The mechanism responsible for the rise in blood pressure in ABT-treated and HET0016-treated rats fed a high salt diet remains to be determined; however, our results are consistent with the hypothesis that inhibition of renal 20-HETE production increases tubular reabsorption of Na^+. In this regard, 20-HETE is normally produced by proximal tubules[3,26] and inhibits Na^+ reabsorption in this nephron segment by inhibiting Na^+/K^+-ATPase activity.[4,31] EETs have also been reported to inhibit Na^+/K^+-ATPase activity,[32] inhibit Na^+ transport in the proximal tubule[33] and rabbit cortical collecting duct,[7] and inhibit vasopressin-stimulated water reabsorption in the collecting duct.[6] 20-HETE also plays a critical role in the regulation of Cl^- transport in the TALH[3,5] and inhibits $Na^+/K^+/2Cl^-$ transport by blocking K^+ channels in the apical membrane of TALH cells,[34] thereby limiting the availability of K^+ for transport via $Na^+/K^+/2Cl^-$ transporters. This reduces the lumen positive transepithelial potential and reduces passive reabsorption of Na^+ in this nephron segment. The findings from the current study, when coupled with these previous observations, suggest that the hypertensive effect observed by lowering renal 20-HETE levels may involve Na^+ retention; however, additional work that demonstrates altered renal Na^+ handling is necessary to accept this hypothesis.

Perspectives

The current study is the first to demonstrate that chronic blockade of the renal formation of 20-HETE increases blood pressure in normally salt-insensitive SD rats fed a high salt diet. The hypertensive effect of 20-HETE inhibitors is salt-dependent, since blood pressure did not increase in rats treated with ABT or HET0016 that were fed a low salt diet. This finding is consistent with the view that the renal formation of 20-HETE influences Na^+ excretion and the regulation of blood pressure during dietary salt loading.

Acknowledgments

This work was supported by grants HL-29574 and HL-36279 from the National Institute of Health. Dr Hoagland was supported by National Research Service Award HL-10364-03. The authors thank Lisa Henderson for valuable technical assistance with the 20-HETE assay and Lynette Jackson for technical assistance with the chronic animal experiments. The authors also thank Taisho Pharmaceutical Company Limited (Saitama, Japan) for providing the HET0016 used in these experiments.

References

1. Roman RJ. P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev.* 2002;82:131–185.
2. Escalante B, Erlij D, Falck JR, McGiff JC. Cytochrome P-450 arachidonate metabolites affect ion fluxes in rabbit medullary thick ascending limb. *Am J Physiol Cell Physiol.* 1994;266:C1775–C1782.
3. Ito O, Roman RJ. Role of 20-HETE in elevating chloride transport in the thick ascending limb of Dahl SS/Jr rats. *Hypertension.* 1999;33:419–423.
4. Schwartzman M, Ferreri NR, Carroll MA, Songu-Mize E, McGiff JC. Renal cytochrome P-450-related arachidonate metabolite inhibits (Na^+ + K^+)ATPase. *Nature.* 1985;314:620–622.
5. Zou AP, Drummond HA, Roman RJ. Role of 20-HETE in elevating loop chloride reabsorption in Dahl SS/Jr rats. *Hypertension.* 1996;27:631–635.
6. Hirt DL, Capdevila JH, Falck JR, Breyer MD, Jacobson HR. Cytochrome P450 metabolites of arachidonic acid are potent inhibitors of vasopressin action on rabbit cortical collecting duct. *J Clin Invest.* 1989;84:1805–1812.
7. Sakairi Y, Jacobson HR, Noland TD, Capdevila JH, Falck JR, Breyer MD. 5,6-EET inhibits ion transport in collecting duct by stimulating endogenous prostaglandin synthesis. *Am J Physiol Renal Fluid Electrolyte Physiol.* 1995;268:F931-F939.
8. Yu Z, Xu F, Huse LM, Morisseau C, Draper AJ, Newman JW, Parker C, Graham L, Engler MM, Hammock BD, Zeldin DC, Kroetz DL. Soluble epoxide hydrolase regulates hydrolysis of vasoactive epoxyeicosatrienoic acids. *Circ Res.* 2000;87:992–998.
9. Imig JD, Zhao X, Capdevila JH, Morisseau C, Hammock BD. Soluble epoxide hydrolase inhibition lowers arterial blood pressure in angiotensin II hypertension. *Hypertension.* 2002;39:690–694.
10. Alonso-Galicia M, Maier KG, Greene AS, Cowley AW Jr, Roman RJ. Role of 20-hydroxyeicosatetraenoic acid in the renal and vasoconstrictor

actions of angiotensin II. *Am J Physiol Regul Integr Comp Physiol.* 2002;283:R60-R68.
11. Xu F, Straub WO, Pak W, Su P, Maier KG, Yu M, Roman RJ, Ortiz De Montellano PR, Kroetz DL. Antihypertensive effect of mechanism-based inhibition of renal arachidonic acid omega-hydroxylase activity. *Am J Physiol Regul Integr Comp Physiol.* 2002;R710-R720.
12. Maier KG, Henderson L, Narayanan J, Alonso-Galicia M, Falck JR, Roman RJ. Fluorescent HPLC assay for 20-HETE and other P-450 metabolites of arachidonic acid. *Am J Physiol Heart Circ Physiol.* 2000; 279:H863–H871.
13. Su, P Kaushal KM, Kroetz DL. Inhibition of renal arachidonic acid omega-hydroxylase activity with ABT reduces blood pressure in the SHR. *Am J Physiol Regul Integr Comp Physiol.* 1998;275:R426–R438.
14. Miyata N, Taniguchi K, Seki T, Ishimoto T, Sato-Watanabe M, Yasuda Y, Doi M, Kametani S, Tomishima Y, Ueki T, Sato M, Kameo K. HET0016, a potent and selective inhibitor of 20-HETE synthesizing enzyme. *Br J Pharmacol.* 2001;133:325–329.
15. Kehl F, Cambj-Sapunar L, Maier KG, Miyata N, Kametani S, Okamoto H, Hudetz AG, Schulte ML, Zagorac D, Harder DR, Roman RJ. 20-HETE contributes to the acute fall in cerebral blood flow after subarachnoid hemorrhage in the rat. *Am J Physiol Heart Circ Physiol.* 2002;282:H1556–H1565.
16. Wang MH, Zhang F, Marji J, Zand BA, Nasjletti A, Laniado-Schwartzman M. CYP4A1 antisense oligonucleotide reduces mesenteric vascular reactivity and blood pressure in SHR. *Am J Physiol Regul Integr Comp Physiol.* 2001;280:R255–R261.
17. Muthalif MM, Benter IF, Khandekar Z, Gaber L, Estes A, Malik S, Parmentier JH, Manne V, Malik KU. Contribution of Ras GTPase/MAP kinase and cytochrome P450 metabolites to deoxycorticosterone-salt-induced hypertension. *Hypertension.* 2000;35:457–463.
18. Oyekan AO, McAward K, Conetta J, Rosenfeld L, McGiff JC. Endothelin-1 and CYP450 arachidonate metabolites interact to promote tissue injury in DOCA-salt hypertension. *Am J Physiol Regul Integr Comp Physiol.* 1999;276:R766–R775.
19. Muthalif MM, Karzoun NA, Gaber L, Khandekar Z, Benter IF, Saeed AE, Parmentier JH, Estes A, Malik KU. Angiotensin II-induced hypertension: contribution of Ras GTPase/Mitogen-activated protein kinase and cytochrome P450 metabolites. *Hypertension.* 2000;36:604–609.
20. Mathews JM, Dostal LA, Bend JR. Inactivation of rabbit pulmonary cytochrome P-450 in microsomes and isolated perfused lungs by the suicide substrate 1-aminobenzotriazole. *J Pharmacol Exp Ther.* 1985; 235:186–190.
21. Mugford CA, Mortillo M, Mico BA, Tarloff JB. 1-Aminobenzotriazole-induced destruction of hepatic and renal cytochromes P-450 in male Sprague-Dawley rats. *Fundam Appl Toxicol.* 1992;19:43–49.
22. Ma YH, Schwartzman ML, Roman RJ. Altered renal P-450 metabolism of arachidonic acid in Dahl salt-sensitive rats. *Am J Physiol Regul Integr Comp Physiol.* 1994;267:R579–R589.
23. Stec DE, Mattson DL, Roman RJ. Inhibition of renal outer medullary 20-HETE production produces hypertension in Lewis rats. *Hypertension.* 1997;29:315–319.
24. Laffer CL, Laniado-Schwartzman M, Wang MH, Nasjletti A, Elijovich F. 20-HETE and furosemide-induced natriuresis in salt-sensitive essential hypertension. *Hypertension.* 2003;41:703–708.
25. Ito O, Roman RJ. Regulation of P-450 4A activity in the glomerulus of the rat. *Am J Physiol Regul Integr Comp Physiol.* 1999;276: R1749–R1757.
26. Omata K, Abraham NG, Schwartzman ML. Renal cytochrome P-450-arachidonic acid metabolism: localization and hormonal regulation in SHR. *Am J Physiol Renal Fluid Electrolye Physiol.* 1992;262: F591–F599.
27. Oyekan AO, Youseff T, Fulton D, Quilley J, McGiff JC. Renal cytochrome P450 ω-hydroxylase and epoxygenase activity are differentially modified by nitric oxide and sodium chloride. *J Clin Invest.* 1999;104: 1131–1137.
28. Stec DE, Trolliet MR, Krieger JE, Jacob HJ, Roman RJ. Renal cytochrome P-4504A activity and salt-sensitivity in SHRs. *Hypertension.* 1996;27:1329–1336.
29. Makita K, Takahashi K, Karara A, Jacobson HR, Falck JR, Capdevila JH. Experimental and/or genetically controlled alterations of the renal microsomal cytochrome P450 epoxygenase induce hypertension in rats fed a high salt diet. *J Clin Invest.* 1994;94:2414–2420.
30. Holla VR, Makita K, Zaphiropoulos PG, Capdevila JH. The kidney cytochrome P-450 2C23 arachidonic acid epoxygenase is upregulated during dietary salt loading. *J Clin Invest.* 1999;104:751–760.
31. Quigley R, Baum M, Reddy KM, Griener JC, Falck JR. Effects of 20-HETE and 19(S)-HETE on rabbit proximal straight tubule volume transport. *Am J Physiol Renal Physiol.* 2000;278:F949–F953.
32. Satoh T, Cohen HT, Katz AI. Intracellular Signaling in the regulation of renal Na-K-ATPase, II: role of eicosanoids. *J Clin Invest.* 1993;91:409–415.
33. Romero, MF, Madhun Z, Hopfer U, Douglas, JG. An epoxygenase metabolite of arachidonic acid 5,6,epoxy-eicosatrienoic acid mediates angiotensin-induced natriuresis in proximal tubule epithelium. In: Sammuelson B, Ramwell P, Paoletti R, Folco G, eds. *Advances in Prostaglandin, Thromboxane and Leukotriene Research.* New York, NY: Raven Press; 1990: 205–208.
34. Wang WH, Lu M, Hebert SC. Cytochrome P-450 metabolites mediate extracellular Ca(2+)-induced inhibition of apical K+ channels in the TAL. *Am J Physiol Cell Physiol.* 1996;271:C103–C111.